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Published in:

Proceedings of the National Academy of Sciences of the United States of America

DOI:

[10.1073/pnas.87.23.9197](https://doi.org/10.1073/pnas.87.23.9197)

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Document Version

Publisher's PDF, also known as Version of record

Publication date:

1990

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Kaay, J. V. D., Draijer, R., & Haastert, P. J. M. V. (1990). Increased conversion of phosphatidylinositol to phosphatidylinositol phosphate in Dictyostelium cells expressing a mutated ras gene. *Proceedings of the National Academy of Sciences of the United States of America*, 87(23), 9197-9201.
<https://doi.org/10.1073/pnas.87.23.9197>

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Increased conversion of phosphatidylinositol to phosphatidylinositol phosphate in *Dictyostelium* cells expressing a mutated *ras* gene

(inositol/cyclic AMP/oncogene/guanine nucleotide-binding protein)

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Communicated by J. T. Bonner, September 4, 1990

ABSTRACT *Dictyostelium discoideum* cells that overexpress a *ras* gene with a Gly¹² → Thr¹² mutation (*Dd-ras-Thr¹²*) have an altered phenotype. These cells were labeled with [³H]inositol and the incorporation of radioactivity into inositol 1,4,5-trisphosphate [Ins(1,4,5)P₃] was analyzed and found to be higher than in control cells. In contrast, the total mass of Ins(1,4,5)P₃, as assessed with an assay using a specific Ins(1,4,5)P₃-binding protein, was not significantly different between control and *Dd-ras-Thr¹²* cells. Cells were labeled with [³H]inositol and the incorporation of radioactivity in all inositol metabolites was analyzed. Increased levels of radioactivity were observed for phosphatidylinositol phosphate (PtdInsP), phosphatidylinositol bisphosphate (PtdInsP₂), Ins(1,4,5)P₃, inositol 1,4-bisphosphate, inositol 4,5-bisphosphate, and inositol 4-monophosphate in *Dd-ras-Thr¹²* cells relative to control cells. Decreased levels were found for phosphatidylinositol (PtdIns) and inositol 1-monophosphate. Calculations on the substrate/product relationships [i.e., Ins(1,4,5)P₃/PtdInsP₂] demonstrate that the observed differences are due only to the increased conversion of PtdIns to PtdInsP; other enzyme reactions, including phospholipase C, are not significantly different between the cell lines. The activity of PtdIns kinase *in vitro* is not different between *Dd-ras-Thr¹²* and control cells, suggesting that either the regulation of this enzyme is altered or that the translocation of substrate from the endoplasmic reticulum to the kinase in the plasma membrane is modified. The results suggest multiple metabolic compartments of Ins(1,4,5)P₃ in *Dictyostelium* cells. In *Dd-ras-Thr¹²* transformants the increased conversion of PtdIns to PtdInsP leads to increased levels of Ins(1,4,5)P₃ in the compartment with a high metabolic turnover. This Ins(1,4,5)P₃ compartment is suggested to be involved in the regulation of cytosolic Ca²⁺ levels.

Mutations in the *ras* gene are often found in cancer cells (1), suggesting that the Ras protein may play a crucial role in the regulation of cell growth and differentiation. The Ras protein is a 21-kDa GTP-binding protein that is highly conserved from yeast and *Dictyostelium* to mammals (2). Its GTP-binding and GTPase activities suggest that the Ras protein belongs to the family of guanine nucleotide-binding proteins (G proteins) that are involved in signal transduction. This supposition is strengthened by the fact that oncogenic mutations result in reduced GTPase activity; in G proteins this would lead to permanent activation and aberrant sensory transduction.

The large family of GTP-binding proteins may be subdivided into two families: (i) heterotrimeric G proteins involved in signal transduction (3) and (ii) monomeric GTP-binding proteins of 18–25 kDa (4). In contrast to signal-transducing G proteins, the small monomeric G proteins are not directly activated by surface receptors, and their regulation is as yet

largely unknown. The protein that activates the GTPase activity in Ras (5, 6) or the protein that increases the dissociation of GDP from Ras (7) could provide the important regulation.

Some members of the family of monomeric GTP-binding proteins are involved in membrane and organelle trafficking (8); the function of the Ras protein, however, is still largely unknown. In yeast, Ras is clearly involved in the regulation of adenylate cyclase activity (9), but this does not appear to be the case in *Dictyostelium* (10) or mammals (11). In these organisms it was suggested that Ras could be the G protein that activates phospholipase C (12, 13). Although the expression of mutated Ras indeed affects D-myo-inositol 1,4,5-trisphosphate [Ins(1,4,5)P₃] levels in many organisms (12–15), the hypothesis that Ras is the signal-transducing G protein that mediates receptor stimulation of phospholipase C has been abandoned (16, 17). The mechanism by which Ras affects Ins(1,4,5)P₃ levels has yet to be demonstrated.

Dictyostelium cells contain two genes that are highly homologous with mammalian *ras*, one gene expressed during growth and one expressed during differentiation (18, 19). Cell lines have been constructed in which the development-related *ras* gene (*Dd-ras*) was overexpressed (10). Overexpression of the wild-type gene has essentially no effect, whereas overexpression of an oncogene-like Gly¹² → Thr¹² mutation leads to an aberrant phenotype (10).

The social amoebae *Dictyostelium* grow on bacteria. Starvation induces cell aggregation that is mediated by chemotaxis to secreted cAMP. The extracellular cAMP signal is transduced by a complex pathway, involving surface receptors, G proteins, and the second messengers cAMP, cGMP, Ins(1,4,5)P₃, and Ca²⁺. The tip of the multicellular structure is thought to be an organizer that regulates cell differentiation yielding only two cell types, dead stalk cells and viable spores (20–22). Mutant cell lines that overexpress the *Dd-ras-Thr¹²* gene form multiple tips in submerged cultures (10). The activation of adenylate cyclase is essentially normal (10, 23), but the cAMP-mediated activation of guanylate cyclase is altered, presumably by an enhanced desensitization (23). Other effects of overexpression of the *Dd-ras-Thr¹²* gene point to the activation of a protein kinase C-like activity (24) and increased levels of Ins(1,4,5)P₃ and inositol hexakisphosphate (InsP₆) in [³H]inositol-labeled cells (13). The most simple conclusion would be that phospholipase C in these cells is activated, yielding increased Ins(1,4,5)P₃ levels; this would lead to elevated Ca²⁺ concentrations, the activation of protein kinase C, and the desensitization of guanylate cyclase, which is known to be inhibited by Ca²⁺ ions (25).

Use of a binding-protein assay to measure absolute Ins(1,4,5)P₃ concentrations in *Dictyostelium* showed that the mass of Ins(1,4,5)P₃ in *Dd-ras-Thr¹²* cells was not different

from that in control cells, although the incorporation of [^3H]inositol into [^3H]Ins(1,4,5) P_3 was increased (26). Similar observations were made for Ins P_6 : [^3H]Ins P_6 levels are increased (13), but the mass of Ins P_6 is not altered (27). This suggests that the effect of overexpression of *Dd-ras-Thr*¹² on [^3H]Ins(1,4,5) P_3 and [^3H]Ins P_6 may be very indirect. Therefore, we have analyzed all inositol metabolites after labeling cells with [^3H]inositol. The results suggest that [^3H]Ins(1,4,5) P_3 levels are increased, not because phospholipase C is activated, but because phosphatidyl inositol (PtdIns) is converted more rapidly to PtdIns P .

MATERIALS AND METHODS

Materials. [^3H]inositol (603 TBq/mmol) and [^3H]Ins(1,4,5) P_3 (1.2 TBq/mmol) were obtained from NEN/DuPont, and [γ - ^{32}P]ATP (37 TBq/mmol) was from Amersham. The Ins(1,4,5) P_3 -binding protein was isolated from bovine adrenal glands (28).

Culture Conditions. *Dictyostelium discoideum* strain AX3 transformed with BS10 vector (control), with vector containing wild-type *ras* (*Dd-ras-Gly*¹²), or with vector containing mutant *ras* (*Dd-ras-Thr*¹²) were grown in HL5 medium supplemented with G418 (Sigma) at 20 $\mu\text{g}/\text{ml}$ as described (10). Cells were harvested at a density of $1\text{--}2 \times 10^6$ per ml and starved for 4 hr at a density of 10^7 per ml in 10 mM sodium/potassium phosphate buffer at pH 6.5 (PB).

Cell Labeling with [^3H]inositol and Analysis of Metabolites. Cells were labeled with [^3H]inositol by electroporation as described (29). In brief, cells were collected, washed by centrifugation, and resuspended at 2×10^8 per ml in PB with [^3H]inositol at 300 $\mu\text{Ci}/\text{ml}$ (1 μCi = 37 kBq). Two high-voltage discharges temporarily permeabilized the cells, allowing highly efficient labeling with [^3H]inositol (29). Cells were then washed three times with PB and resuspended at 10^7 per ml in PB. Aliquots were taken after 30–60 min and centrifuged, and cells were lysed by a mixture containing 9% (wt/vol) trichloroacetic acid, 5 mM EDTA, and 50 μg of phytic acid hydrolysate per ml (final concentrations). Samples were centrifuged and the supernatant was analyzed by HPLC on a reversed-phase ion-pair system and on a Partisil SAX column as described (29). The pellets were extracted with 500 μl of chloroform/methanol/concentrated hydrochloric acid, 20:40:1 (vol/vol). After the addition of 200 μl of H_2O to induce phase separation, the organic phase was processed for the analysis of phospholipids by TLC on Silica 60 plates that were developed in chloroform/methanol/concentrated ammonium hydroxide/water, 90:90:5:22. Radioactive spots were visualized by EN³HANCE spray (NEN) and fluorography, followed by scraping of the spots and quantitative determination by scintillation counting.

PtdIns Kinase. Cells were washed once in PB, once in 136 mM Tris/HCl at pH 7.6 (buffer A), and resuspended at 10^8 per ml in this buffer. Cells were lysed by rapid passage through a Nuclepore filter (pore size, 3 μm). The kinase reaction mixture (100 μl) contained buffer A, 1 mM MgCl_2 , 10 mM NaF, 25 mM KCl, 0.1 mM ATP, 1 μCi of [γ - ^{32}P]ATP, and 20 μl of homogenate (30). After incubation for 0, 30, 60, or 90 sec the reaction was quenched by the addition of 500 μl of

chloroform/methanol/concentrated hydrochloric acid, 20:40:1. Phase separation was induced by the addition of 200 μl of H_2O ; the organic phase was washed with 200 μl of 2 M KCl, dried by a stream of nitrogen, and resuspended in 50 μl of chloroform/methanol, 9:1. The samples were chromatographed on Silica 60 plates in chloroform/methanol/concentrated ammonium hydroxide/water, 90:90:5:22. The plates were exposed to Fuji x-ray film and the radioactivity in the spots was determined by scintillation counting.

RESULTS

Europe-Finner *et al.* (13) have demonstrated that *Dictyostelium* cells expressing a mutated *ras* gene contain higher levels of [^3H]Ins(1,4,5) P_3 than control cells after metabolic labeling with [^3H]inositol. We observed (29) that not all of this radioactivity may have been [^3H]Ins(1,4,5) P_3 ; therefore, we reanalyzed the water-soluble fraction of [^3H]inositol-labeled cells by reversed-phase ion-pair HPLC. The results are in agreement with previous data that *Dd-ras-Thr*¹² cells contain elevated [^3H]Ins(1,4,5) P_3 levels (Table 1). Since previous data also indicated increased levels of [^3H]Ins P_6 (13) without an increase of the absolute Ins P_6 concentration (27), the mass of Ins(1,4,5) P_3 was determined by a sensitive assay using an Ins(1,4,5) P_3 -binding protein (26). The absolute Ins(1,4,5) P_3 concentration was not significantly different among the three cell lines (Table 1). Radioactive inositol apparently is metabolized more actively to Ins(1,4,5) P_3 and Ins P_6 in *Dd-ras-Thr*¹² cells, without significant effects on their respective masses. Therefore, all inositol-containing metabolites were analyzed after a short labeling period with [^3H]inositol.

Cells were briefly labeled with [^3H]inositol and incubated for 30–60 min to allow the [^3H]inositol to be metabolized. Then, cells were extracted, and the phospholipids were analyzed by TLC and the water-soluble inositol phosphates by ion-exchange and ion-pair HPLC (29) (Table 2). The uptake of [^3H]inositol was not significantly different among the three cell lines (data not shown). However, the distribution among the various metabolites was quite different, with the transformant overexpressing the wild-type gene showing data that were usually between those of control cells and those of the transformant overexpressing the mutated gene; we will describe the data of *Dd-ras-Thr*¹² relative to control cells. The radioactivities of PtdIns and Ins $1P$ were significantly lower in *Dd-ras-Thr*¹² cells than in control cells. In contrast, the radioactivities of PtdIns P , PtdIns P_2 , Ins $4P$, Ins(1,4) P_2 , Ins(4,5) P_2 , and Ins(1,4,5) P_3 were all significantly higher in *Dd-ras-Thr*¹² cells than in control. Fig. 1 shows a fluorogram of [^3H]inositol-containing phospholipids, demonstrating the increased labeling of PtdIns P and PtdIns P_2 . We also observed several unidentified spots that are tentatively addressed as lyso-PtdIns and PtdInsY; the labeling of these spots was not significantly different between control and *Dd-ras-Thr*¹² cells (Table 2). The observation that the increased incorporation of radioactivity is not restricted to Ins(1,4,5) P_3 but also present in inositolphospholipids suggests that the expression of mutated *ras* may affect an enzyme in the inositol cycle other than phospholipase C.

Table 1. Mass and radioactivity of Ins(1,4,5) P_3 per 10^7 cells

Quantity	Value		
	AX3	<i>Dd-ras-Gly</i> ¹²	<i>Dd-ras-Thr</i> ¹²
Ins(1,4,5) P_3 mass, pmol	18.3 ± 2.0	15.8 ± 2.4	14.8 ± 1.8
Ins(1,4,5) P_3 label, cpm	475 ± 33	$713 \pm 150^*$	$735 \pm 171^*$
Inositol label, cpm	$30,350 \pm 2150$	$33,875 \pm 2200$	$32,100 \pm 1850$

The mass of Ins(1,4,5) P_3 was determined by a binding-protein assay (26). The radioactivity of [^3H]Ins(1,4,5) P_3 was determined after labeling of the cells with 15 μCi of [^3H]inositol and extraction of the water-soluble products. The analysis was done by reversed-phase ion-pair HPLC.

*, Significantly different from control (AX3) at $P > 0.05$ ($n = 4$).

Table 2. [^3H]inositol metabolites in control and *ras*-transformed cells

Compound	Fraction of incorporated radioactivity, %		
	AX3	<i>Dd-ras-Gly</i> ¹²	<i>Dd-ras-Thr</i> ¹²
Inositol	23.02 \pm 1.64	23.26 \pm 1.51 (101 \pm 10, NS)	25.42 \pm 1.46 (110 \pm 10, NS)
GroPIIns	0.32 \pm 0.03	0.28 \pm 0.02 (88 \pm 10, NS)	0.43 \pm 0.07 (134 \pm 25, *)
Ins1P	2.51 \pm 0.27	2.13 \pm 0.30 (85 \pm 15, NS)	1.59 \pm 0.40 (63 \pm 17, †)
Ins4P	0.50 \pm 0.07	0.61 \pm 0.09 (122 \pm 25, NS)	0.86 \pm 0.12 (172 \pm 34, †)
Ins(1,4)P ₂	0.55 \pm 0.12	1.00 \pm 0.14 (180 \pm 47, *)	1.10 \pm 0.14 (200 \pm 51, †)
Ins(4,5)P ₂	0.44 \pm 0.09	0.63 \pm 0.12 (143 \pm 40, NS)	0.76 \pm 0.16 (173 \pm 51, *)
Ins(1,4,5)P ₃	0.36 \pm 0.07	0.54 \pm 0.12 (151 \pm 44, *)	0.58 \pm 0.13 (162 \pm 48, *)
InsP _x	13.58 \pm 0.49	15.58 \pm 0.52 (115 \pm 6, †)	18.05 \pm 1.37 (133 \pm 11, †)
PtdIns	39.40 \pm 0.63	34.21 \pm 0.93 (87 \pm 3, ‡)	31.11 \pm 1.13 (79 \pm 3, ‡)
PtdInsP	1.22 \pm 0.17	2.01 \pm 0.35 (165 \pm 37, *)	2.73 \pm 0.19 (224 \pm 35, ‡)
PtdInsP ₂	1.44 \pm 0.17	1.89 \pm 0.47 (131 \pm 36, *)	2.47 \pm 0.21 (171 \pm 25, ‡)
"Lyso-PtdIns"	3.40 \pm 1.16	4.70 \pm 1.32 (138 \pm 61, NS)	3.48 \pm 2.09 (102 \pm 70, NS)
PtdInsY	3.00 \pm 1.16	2.27 \pm 0.79 (76 \pm 40, NS)	2.61 \pm 1.02 (87 \pm 48, NS)

Cells were labeled with 15 μCi per sample, incubated for 30–60 min, and extracted for the analysis of water-soluble products by HPLC and phospholipids by TLC. InsP_x denotes the unidentified peak that is eluted at the end of the reversed-phase HPLC column (29) and could be InsP₆ (13). "Lyso-PtdIns" and PtdInsY are spots on the TLC chromatogram that are tentatively or not identified (see Fig. 1). Gro, glycerol. The radioactivity in the various compounds was determined and the percentage of the total was calculated. In parentheses, the radioactivity in each compound of the transformed cells is given as a percentage of the radioactivity in the same compound of control cells; NS, not significantly different from 100%; *, †, and ‡, significantly different from 100% at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively (paired t test with $n = 4$).

The relationship between substrate and product was calculated for each step of the inositol cycle in *Dictyostelium*. The data should not be used to calculate relative enzyme activities, because each metabolite takes part in at least two enzyme reactions; however, this method may provide evidence for the enzyme reaction that is most strongly altered by the *ras* mutation. The inositol cycle that was used for this purpose (Fig. 2) was derived from combining experiments on the metabolism of Ins(1,4,5)P₃ *in vitro* (31) with experiments on the dynamics by which these compounds are labeled after

a pulse-chase with [^3H]inositol *in vivo* (unpublished observations). The calculations show (Table 3 and Fig. 2) that the PtdInsP₂/Ins(1,4,5)P₃ ratio is not altered in *Dd-ras-Thr*¹² cells relative to control cells, suggesting that phospholipase C activity is not strongly affected by overexpression of the mutated *ras* gene. The same conclusion is reached for all other reactions, with the exception of the conversion of PtdIns to PtdInsP, which appears to be about 3-fold faster in *Dd-ras-Thr*¹² cells than in control cells. These data strongly suggest that [^3H]Ins(1,4,5)P₃ levels are higher because PtdInsP₂ levels are higher, which is due to elevated levels of [^3H]PtdInsP. The situation for the inositol monophosphate is especially instructive: Ins1P levels are decreased because [^3H]PtdIns is low, whereas Ins4P levels are increased because radioactivities of Ins(1,4)P₂ and Ins(4,5)P₂ are elevated. Thus, the effect of overexpression of a mutated *ras*

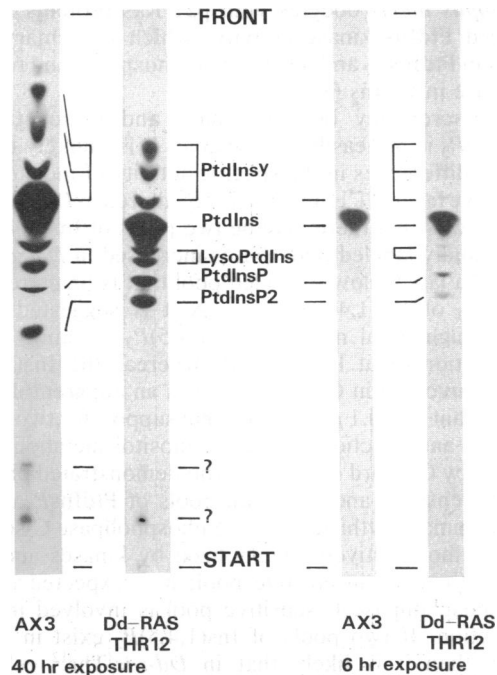


FIG. 1. TLC of inositolphospholipids extracted from control (AX3) and *Dd-ras-Thr*¹² cells. Cells were labeled with [^3H]inositol, and phospholipids were extracted, separated by TLC, and visualized by fluorography. Spots were identified by cochromatography with authentic standards. Several unidentified spots are indicated as lyso-PtdIns and PtdInsY. Two exposure times (6 and 40 hr) are shown.

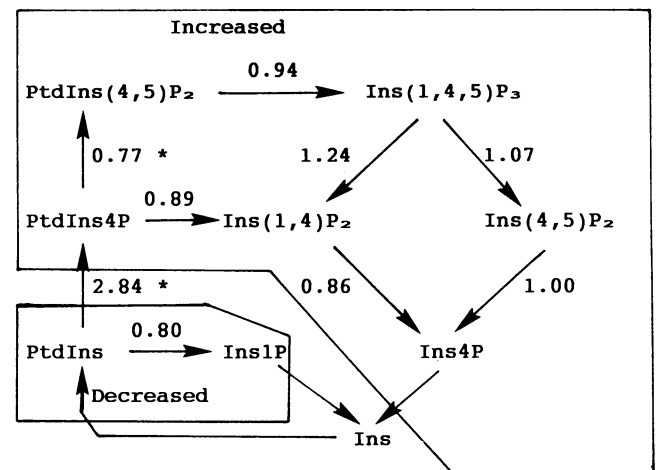


FIG. 2. Inositol cycle of *D. discoideum*. The inositol cycle of *Dictyostelium* was unraveled by analysis of Ins(1,4,5)P₃ degradation *in vitro* and of [^3H]inositol metabolites after a pulse-chase experiment (29, 31). The radioactivity in the metabolites is increased or decreased in *Dd-ras-Thr*¹² cells relative to control cells. Numbers indicate the product/substrate ratio of the reaction of *Dd-ras-Thr*¹² cells relative to this ratio in control cells; starred values are significantly different from control. See Tables 2 and 3 for primary data.

Table 3. Balance of inositol phosphates

Substrate	Product	Product/substrate ratio (% of AX3)		
		AX3	<i>Dd-ras</i> -Gly ¹²	<i>Dd-ras</i> -Thr ¹²
PtdIns	Ins1P	0.064 ± 0.007	0.062 ± 0.009 (98 ± 18, NS)	0.051 ± 0.013 (80 ± 20, NS)
PtdInsP	Ins(1,4)P ₂	0.451 ± 0.117	0.498 ± 0.111 (110 ± 38, NS)	0.403 ± 0.058 (89 ± 26, NS)
PtdInsP ₂	Ins(1,4,5)P ₃	0.250 ± 0.057	0.270 ± 0.100 (108 ± 47, NS)	0.296 ± 0.058 (119 ± 36, NS)
Ins(1,4,5)P ₃	Ins(1,4)P ₂	1.528 ± 0.446	1.961 ± 0.604 (128 ± 54, NS)	1.507 ± 0.330 (99 ± 36, NS)
Ins(1,4,5)P ₃	Ins(4,5)P ₂	1.222 ± 0.345	1.235 ± 0.413 (101 ± 44, NS)	1.041 ± 0.283 (86 ± 34, NS)
Ins(1,4)P ₂	Ins4P	0.909 ± 0.236	0.610 ± 0.124 (67 ± 22, *)	0.782 ± 0.147 (86 ± 27, NS)
Ins(4,5)P ₂	Ins4P	1.136 ± 0.281	0.968 ± 0.233 (85 ± 29, NS)	1.132 ± 0.286 (100 ± 35, NS)
Ins1P	Ins	9.17 ± 1.18	10.92 ± 1.69 (119 ± 24, NS)	15.99 ± 4.33 (174 ± 52, *)
Ins4P	Ins	46.04 ± 7.23	38.13 ± 7.10 (83 ± 20, NS)	29.56 ± 4.46 (64 ± 14, †)
Ins	PtdIns	1.712 ± 0.125	1.471 ± 0.104 (86 ± 9, *)	1.224 ± 0.083 (72 ± 7, ‡)
PtdIns	PtdInsP	0.031 ± 0.004	0.059 ± 0.010 (190 ± 40, †)	0.088 ± 0.007 (284 ± 43, ‡)
PtdInsP	PtdInsP ₂	1.180 ± 0.216	0.940 ± 0.285 (80 ± 28, NS)	0.905 ± 0.099 (77 ± 16, *)

The ratio of radioactivity in substrate and product was calculated for each step of the inositol cycle that has been demonstrated to exist. Although this calculation cannot be used as a measure of enzyme activity, it allows one to identify the enzyme reaction that is different in the *Dd-ras*-Thr¹² cells. See Table 2 for original data and Fig. 2 for the inositol cycle in *Dictyostelium*. NS, not significantly different from 100%; *, †, and ‡, significantly different from 100% at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively (paired *t* test with $n = 4$).

gene on the inositol cycle can be traced to the conversion of PtdIns to PtdInsP.

The activity of PtdIns kinase (as well as PtdInsP kinase) is demonstrated in Fig. 3, showing the phosphorylation of membrane phospholipids in a cell-free system. The results show no significant difference among the three cell lines that contain the transformation vector, suggesting that overexpression of the wild-type or mutant *ras* gene apparently has no effect on the activity of PtdIns kinase.

DISCUSSION

The balance of [³H]inositol metabolites in *Dictyostelium* cells expressing a mutant *ras* oncogene was analyzed because previous data demonstrated that radioactivity of InsP₆ was increased whereas the mass of InsP₆ was not changed in these *Dd-ras*-Thr¹² cells (13, 27). We made a similar observation for

Ins(1,4,5)P₃; the mass was not altered, but more radioactivity was found in this compound after *Dd-ras*-Thr¹² cells were labeled with [³H]inositol. The balance of inositol metabolites demonstrates that the underlying mechanism of increased [³H]Ins(1,4,5)P₃ levels is not provided by the enzymes that synthesize or degrade this compound but is provided by a more distal reaction, the conversion of PtdIns to PtdInsP. The relative incorporation of radioactivity in all other inositol metabolites can be accounted for by this increased conversion. Thus, there is no reason to propose that increased levels of [³H]Ins(1,4,5)P₃ are due to increased activity of phospholipase C. Other reports confirm a possible role of Ras in the regulation of PtdIns kinase activity. Huang *et al.* (32) found that PtdIns and PtdInsP kinase activities are increased in membranes from *ras*-transformed fibroblasts. Microinjection of *Xenopus laevis* oocytes with p21 Ras proteins leads to enhanced PtdIns kinase activity, which is primary to increases in PtdInsP₂ and inositol polyphosphate and results in a decrease in PtdIns (33).

The discrepancy between mass and radioactivity in Ins(1,4,5)P₃ is not easily explainable. It is unlikely that this is due to differences in the specific activity of inositol, since the relative level of [³H]inositol is not altered in *Dd-ras*-Thr¹². This suggests that there may be two pools of Ins(1,4,5)P₃; a small, rapidly labeled pool that is increased in *Dd-ras*-Thr¹² cells and a large, slowly labeled pool that is unaltered. That two pools of Ins(1,4,5)P₃ may exist is suggested by the extreme high basal mass of Ins(1,4,5)P₃, yielding a mean concentration of at least 3 μ M, whereas the Ins(1,4,5)P₃ receptor involved in Ca²⁺ release has an apparent dissociation constant of <0.1 μ M. Additional support for two distinct metabolic and functional pools of inositol metabolites was provided by Gascard *et al.* (34), who demonstrated phospholipase C-sensitive and -resistant pools of PtdInsP and PtdInsP₂ in human erythrocytes. The phospholipase C-sensitive pool was more actively metabolized by kinases and phosphatases than the insensitive pool; it is expected that the small phospholipase C-sensitive pool is involved in signal transduction. If two pools of Ins(1,4,5)P₃ exist in *Dictyostelium*, then it is likely that in *Dd-ras*-Thr¹² cells, the Ins(1,4,5)P₃ concentration is actually elevated in the rapidly labeling responsive pool and that increased levels of Ca²⁺ or protein kinase C could be present even though the total Ins(1,4,5)P₃ mass in the cell is not altered. This hypothesis is supported by previous observations suggesting elevated levels of Ca²⁺ and protein kinase C activity in *Dd-ras*-Thr¹² transformants (23, 24).

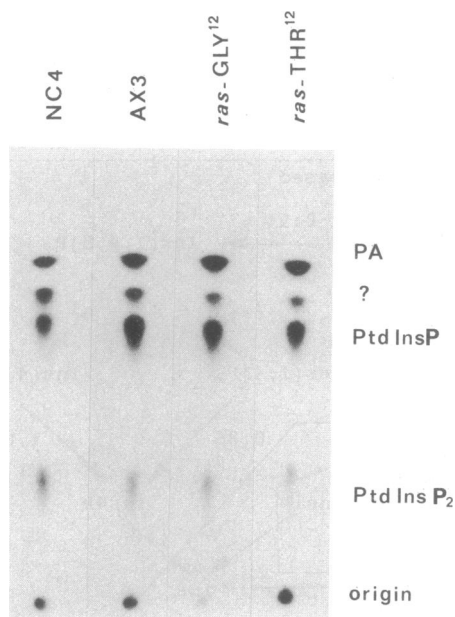


FIG. 3. PtdIns kinase activity. Membranes from control cells (AX3) and from cells overexpressing the wild-type (*Dd-ras*-Gly¹²) or mutant (*Dd-ras*-Thr¹²) gene were incubated with [³²P]ATP. The phospholipids were analyzed by TLC, and the compounds were identified by cochromatography with authentic standards. PA, phosphatidic acid. Membranes from the bacterially grown wild-type strain NC4 were also used in this experiment for comparison with previous experiments (30).

Several explanations can account for the enhanced conversion of PtdIns to PtdInsP. Altered conformation and/or levels of the PtdIns kinase seem unlikely because we have not found differences in enzyme activities *in vitro* among the three cell lines. For the same reason, direct regulation of the enzyme by Ras seems unlikely. In mammalian cells the PtdIns kinase is present predominantly in the plasma membrane, whereas PtdIns is synthesized mainly in the endoplasmic reticulum. Therefore, increased PtdInsP formation in *ras*-transformed cells could be due to increased substrate availability if PtdIns is translocated more actively from the endoplasmic reticulum to the plasma membrane. Alternatively, in mammalian cells PtdIns kinase activities have been found in both the endoplasmic reticulum and the plasma membrane (35), again suggesting the existence of two independent metabolic inositol phospholipid pools. Therefore, in *ras*-transformed cells there could be increased PtdIns kinase activity or substrate availability in only one of these metabolic pools.

For the inositol cycle in *D. discoideum* we have tentatively identified the principal effect of mutated *ras* as the increased conversion of PtdIns to PtdInsP. This conversion is a relatively complex reaction and has not been well analyzed in *Dictyostelium*. The mechanism by which Ras proteins interfere with the formation of PtdInsP needs further investigation. The present results imply that different metabolic and functional pools of inositolphospholipids have to be considered.

This study was started at the Department of Cell Biology and Genetics of the University of Leiden; we thank Anthony Bominaar, Fanja Kesbeke, Ewa Snaar-Jagalska, and Pauline Schaap for stimulating discussions. We gratefully acknowledge Eva Luderus and Christophe Reymond for providing the cells. This study was supported by the C. and C. Huygens Fund, which is subsidized by the Netherlands Organization of Research, and by a grant from the Langerhuizen Fund.

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